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GLUTATHIONE-DEPENDENT PROTECTION BY RAT LIVER MICROSOMAL PROTEIN AGAINST LIPID PEROXIDATION

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GSH is an important cellular defense against oxidant injury. Its effect in the rat liver microsomal lipid peroxidation system has been examined. Incubation of fresh rat liver microsomes with ascorbic acid and ADP-chelated iron leads to the peroxidation of microsomal lipids (production of thiobarbituric acid-reactive substances and destruction of polyunsaturated fatty acids) following a 2 to 5 min lag. Addition of 0.1 mM GSH to the system lengthened the lag period by 5 to 15 min without affecting the rate or the extent of lipid peroxidation. GSH could not be replaced in prolonging the lag by cysteine, mercaptoethanol, dithiothreitol, propylthiouracil, or GSSG. The GSH effect on the lag was abolished by heating or trypsin digestion of the microsomes, indicating that microsomal protein is required for its expression. Progressively longer lags were observed as the GSH concentration was increased from 0.1 to 5 mM, but there was no evidence of GSH oxidation as a consequence of the protection against lipid peroxidation. GSH protected against heat inactivation of the microsomal protein responsible for the GSH effect. Experiments with an oxygen electrode revealed that the GSH protection did not alter the ratio of O_2 consumed to thiobarbituric acid-reactive substances produced. This implicated free radical scavenging as the mechanism of protection. These results indicate the existence of a GSH-dependent rat liver microsomal protein which scavenges free radicals. This protein may be an important defense against free radical injury to the microsomal membrane.

Introduction

Cellular oxidant defenses are necessary to prevent peroxidation of membrane polyunsaturated fatty acids by free radicals and oxygen present in all animal cells. GSH, acting through enzymes utilizing it as a substrate or cofactor, is thought to be an important oxidant defense. Just how it accomplishes this task, however, has not been fully elucidated.

Several years ago McCay et al. [1] noted that simultaneous addition of GSH and dialyzed rat liver $105\,000 \times g$ supernatant to the rat liver microsomal lipid peroxidation system blocked lipid peroxidation. Later Burk et al. [2] and Gibson et al. [3] showed that the GSH-dependent protective activity of the $105\,000 \times g$ supernatant was not due to selenium-dependent glutathione peroxidase but to other proteins.

In the course of studies of those $105\,000 \times g$ supernatant proteins, we noted that low concentrations of GSH alone delayed the onset of lipid peroxidation in the microsomal lipid peroxidation system [4]. We have characterized this protective effect of GSH against lipid peroxidation and find that it requires microsomal protein. This mechanism appears to be well-suited for protecting the microsomal membrane against lipid peroxidation

and may be an important in vivo oxidant defense. Part of this work has been published in abstract form [5].

Methods

Microsomal preparation and incubation. Male Holtzman rats were fed a nutritionally adequate semisynthetic diet from weaning. The diet contained 100 IU of vitamin E per kg as α-tocopheryl acetate, 0.5 mg selenium per kg as Na_2SeO_3, and 6.7% corn oil [6]. Liver microsomes were isolated from fed rats weighing 350–500 g and washed once in 0.15 M KCl as described previously [2]. They were suspended at a concentration of approx. 10 mg protein per ml. Boiled microsomes were prepared by heating a test tube containing fresh microsomes in a boiling water bath for 1 min.

The incubations depicted in Figs. 1 and 3–7 were carried out at 37°C in a shaking water bath in open 25 ml flasks. The buffer used was 50 mM Tris-HCl, pH 7.5, containing 0.14 M NaCl. This buffer is referred to as the incubation buffer. It was used for final suspension of microsomes and preparation of incubation reagents. Microsomal protein concentration in the incubation was approx. 0.5 mg/ml. Other final concentrations were: 0.5 mM L-ascorbic acid, 2 mM ADP, 6 μM $FeCl_3$. Incubation volume was 5 ml. The ADP and $FeCl_3$ were combined and allowed to stand at room temperature for 1 h before use. In the experiment shown in Fig. 8, 300 μM NADPH was substituted for ascorbate.

The incubations carried out in the oxygen electrode (Fig. 8) and in the cuvette (Fig. 9) were scaled down in volume but had the same concentrations of constituents as those described above. Also they were stirred instead of shaken. The Clark-type oxygen electrode was purchased from the Johnson Foundation, (Philadelphia, PA). It was calibrated with buffer containing 187 μM O_2 and sodium dithionite. The dual wavelength experiments were carried out in an Aminco DW-2a spectrophotometer (American Instrument Co., Silver Spring, MD).

The incubations carried out in the experiment shown in Fig. 2 utilized the same concentrations of constituents described above but had a volume of 20 ml and were done in 50 ml flasks. The reaction was stopped at the appropriate time by the addition of 1 ml of 20 mM disodium EDTA in incubation buffer. Thiobarbituric acid-reactive substances were determined and the microsomes were sedimented by centrifugation at 105 000 × *g* for 60 min. They were resuspended in 1 ml of incubation buffer and the lipids were extracted by the method of Bligh and Dyer [7]. Heptadecanoic acid was added as an internal standard. The lipid extract was saponified with 0.5 M NaOH in methanol for 60 min at 70°C. Then it was methylated with 14% BF_3 in methanol for 5 min at 75°C. After petroleum ether extraction, the methylated fatty acids were taken up in heptane and analysis was carried out in a Hewlett-Packard 5880 A gas chromatography apparatus with a flame-ionization detector. The column used was SP2330 Chromasorb (100/120) from Supelco (Bellafonte, PA) Oven temperature was programmed from 160 to 230°C at 30°C/min. Calculations were made with reference to the internal standard. Data are presented as percent of the initial values.

For the sonication and washing experiment, microsomes were prepared as usual and split into two aliquots which were suspended in the incubation buffer containing 1 mM GSH. One aliquot was kept on ice and the other was sonicated for 30 s (60 s on 50% cycle) on full power setting (200 W) of a Heat Systems Model W-225-R sonicator using the cup horn (Heat Systems-Ultrasonics, Inc., Plainview, NY). The microsomes were sedimented in the ultracentrifuge, then resuspended in the same buffer and sedimented again. From this point the iced or control microsomal aliquot and the sonicated aliquot were treated identically. They were twice suspended in incubation buffer without GSH and sedimented ultracentrifugally to remove GSH. Then they were used for the standard incubation procedure in which the lag phase was determined without GSH and with 0.1 mM GSH.

Trypsin digestion of the microsomes was carried out as follows: microsomes were suspended to a concentration of 2 mg protein/ml in 50 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$. They were incubated for 25 min at 30°C with 0.3 mg of Sigma type III trypsin per ml. The incubation was stopped by the addition of 0.6 mg of soybean trypsin inhibitor per

ml of digestate and cooling the flasks on ice. The microsomes were sedimented by ultracentrifugation, resuspended in 0.15 M KCl and resedimented. They were then resuspended in the microsomal lipid peroxidation incubation buffer and used for experiments the same day. The control microsomes were subjected to the same treatment except no trypsin was added to them.

Liver 105 000 × *g* supernatant was prepared by homogenizing a liver in the incubation buffer at 25% weight volume and centrifuging it at 105 000 × *g* for 60 min. The resulting supernatant was dialyzed overnight against incubation buffer at 4°C to remove glutathione before it was used in incubation experiments.

Assays. Thiobarbituric acid-reactive substances were assayed as described previously and results were expressed in malonaldehyde equivalents using malonaldehyde bis(dimethyl acetal) as standard [2,8]. The assay mixture included 0.1% butylated hydroxytoluene to prevent the formation of thiobarbituric acid-reactive substances during the assay procedure. GSH was determined with 5,5′-dithiobis-(2-nitrobenzoic acid) [9,10]. Protein was determined by the method of Lowry et al. [11] using bovine serum albumin as a standard.

Materials. Trypsin (type III), soybean trypsin inhibitor, L-ascorbic acid, ADP, GSH, GSSG, thiobarbituric acid, butylated hydroxytoluene, dithiothreitol, and propylthiouracil were purchased from Sigma Chemical Co., St. Louis, MO. 2-mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, NY. Aldrich Chemical Co., Milwaukee, WI supplied the 5,5′-dithiobis-(2-nitrobenzoic acid) and the malonaldehyde bis(dimethyl acetal). Boehringer Mannheim, Indianapolis, IN, supplied the NADPH.

Results

Inhibition of microsomal lipid peroxidation by GSH

Incubation of rat liver microsomes with iron and ascorbate leads to rapid peroxidation of the microsomal lipids following a lag of several minutes. GSH delays the onset of lipid peroxidation in this system when fresh microsomes are used. Fig. 1 demonstrates that the 4 min lag found in the absence of GSH could be lengthened to 15 min by the addition of 0.1 mM GSH to the flasks.

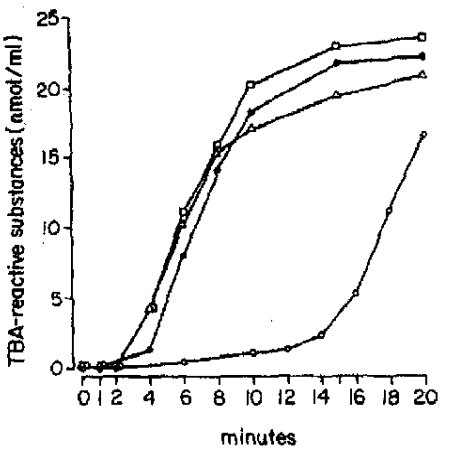

Fig. 1. Effect of GSH on ascorbate-iron lipid peroxidation in fresh and boiled microsomes. The incubations done were: ●———●, fresh microsomes with no GSH; ○———○, fresh microsomes with 0.1 mM GSH; △———△, boiled microsomes with no GSH; □———□, boiled microsomes with 0.1 mM GSH. TBA, thiobarbituric acid.

GSH had no effect on the rate or extent of lipid peroxidation, however.

Although measurement of thiobarbituric acid-reactive substances is a standard assay for lipid peroxidation, it is theoretically possible that GSH could merely be causing the elimination of the thiobarbituric acid-reactive substances rather than preventing lipid peroxidation. Therefore we measured the individual fatty acids in the microsomes and correlated them with lipid peroxidation as measured by thiobarbituric acid-reactive substance determination. In Fig. 2A is shown the result when no GSH is present. The rise in thiobarbituric acid-reactive substances is accompanied by a striking fall in the concentration of the polyunsaturated fatty acids 20 : 4 and 22 : 6. Fig. 2B demonstrates that GSH not only blocks thiobarbituric acid-reactive substance formation but also prevents 20 : 4 and 22 : 6 destruction. Thus, GSH is preventing lipid peroxidation.

Requirement of a microsomal protein for the GSH protection

This protection by GSH requires another factor. Heated microsomes showed no GSH effect (Fig. 1). Trypsinization of the microsomes (Fig. 3) also abolished the GSH effect, and even eliminated the short lag seen in untreated microsomes in the

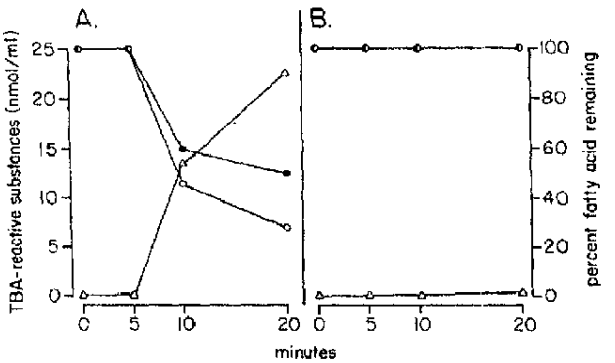

Fig. 2. Correlation of thiobarbituric acid-substance production with polyunsaturated fatty acid consumption and prevention of both by GSH. No GSH was added in the panel A experiments. The panel B experiments contained 0.1 mM GSH. Measurements shown are: △———△, thiobarbituric acid-reactive substances; ●———●, 20:4 fatty acid; ○———○, 22:6 fatty acid. Other fatty acids measured which showed no decrease in either experiment were 16:0, 18:0, and 18:1. 18:2 was also measured and showed a 15% decrease at 20 min when no GSH was present. No decrease in 18:2 was noted in the presence of GSH.

absence of GSH. These results strongly suggest that the protective factor mediating the GSH effect is a protein.

Several experiments were performed to determine whether the protective factor is bound to the microsomal membrane or is a contaminant. Washing the microsomal fraction by the method of Weihing et al. [12] to the point where no lactic dehydrogenase [13] was detectable failed to abolish the GSH effect. Sonication and then washing by centrifugation as described in Methods also had no affect on the length of the lag caused by GSH. In another experiment microsomes were heated to inactivate the protective factor and were then resuspended in fresh 105 000 × *g* liver supernatant and reisolated as originally done. No GSH protective effect was observed with these microsomes, suggesting that contamination by a cytosolic factor during isolation is not responsible for the GSH effect.

Finally, an experiment was done to simulate heavy contamination of the microsomal fraction by 105 000 × *g* supernatant. Boiled microsomes were used. The effect of 0.5 ml of dialyzed 105 000 × *g* liver supernatant on lipid peroxidation was assessed in the presence of 0.1 mM GSH and in its absence. The 105 000 × *g* supernatant had no effect in either case. These four experiments indicate that the protective factor is not a contaminant of the microsomal fraction from the 105 000 × *g* supernatant.

The experiment shown in Fig. 4 demonstrates how closely the factor is associated with the membrane. Incubation of a mixture of heated and fresh microsomes in the presence of GSH resulted in the same amount of lipid peroxidation as was observed in the fractions when they were incubated separately and the results were summed. This indi-

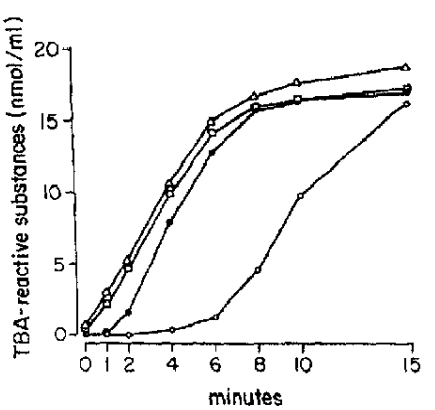

Fig. 3. Effect of trypsin digestion of microsomes on the GSH protection against ascorbate-iron lipid peroxidation. Treatments were: ●———●, control with no GSH; ○———○, control with 0.1 mM GSH; △———△, trypsin-digested with no GSH; □———□, trypsin-digested with 0.1 mM GSH.

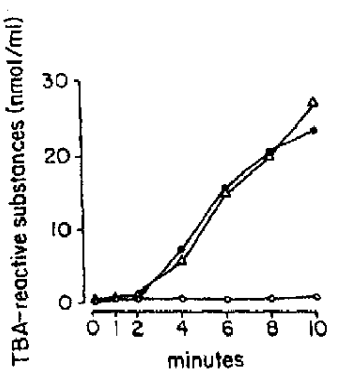

Fig. 4. Effect of mixing boiled and fresh microsomes on GSH protection against ascorbate-iron lipid peroxidation. All flasks contained 0.1 mM GSH and differed only in microsomes added: ○———○, fresh microsomes (0.5 mg protein/ml); ●———●, boiled microsomes (0.5 mg protein/ml); △———△, boiled and fresh microsomes (0.5 mg protein/ml of each).

cates that the boiled microsomes could not be protected by the fresh ones in the mixture, but that the fresh microsomes were protected by GSH even in the presence of the peroxidizing boiled microsomes. This suggests that the protective factor does not readily transfer from one membrane to another.

Specificity for GSH and role of GSH in protection

The protective effect is highly specific for GSH. Addition of 0.1 or 0.5 mM cysteine, mercaptoethanol, propylthiouracil, or dithiothreitol, had no effect on the length of the lag (data not shown). Oxidation of GSH rendered it inactive in this system. GSSG added at 0.1 or 0.5 mM had no effect on the lag (data not shown).

Several experiments were carried out to characterize the role of GSH in protecting against microsomal lipid peroxidation. Raising GSH concentration lengthened the lag before rapid lipid peroxidation occurred. Significant prolongation of the lag was caused by GSH concentrations as low as 0.02 mM. In an experiment comparing the effect of different concentrations of GSH on the lag, 0, 0.1, 1.0, and 5.0 mM GSH gave lag times of 2, 11, 29 and 37 min, respectively. However, once rapid lipid peroxidation started, the GSH concentration did not affect its rate or the total thiobarbituric acid-reactive substances produced (approx. 40 nmol/ml in this experiment). Thus, even though increases in GSH concentration lead to increases in the lag, the increase in the lag is not directly proportional to the GSH added.

The experiment shown in Fig. 5 was designed to determine whether GSH is oxidized as a consequence of its protective action. A flask with fresh microsomes and another flask with heated microsomes, both containing added GSH, were incubated and thiobarbituric acid-reactive substances as well as GSH were measured at intervals. The fresh microsomes were protected against lipid peroxidation by GSH but caused no more loss of GSH (33%) than did the heated microsomes (47%), which were not protected. In an identical incubation with microsomes omitted GSH loss was 8%. Furthermore, the protective lag in the fresh microsomes ended between 20 and 30 min while there was still a large amount of GSH present. This suggests that the protective effect of GSH is not

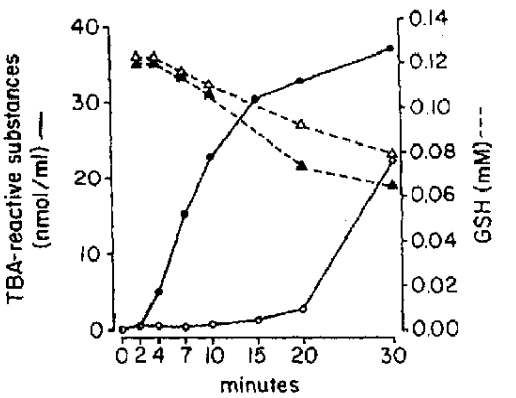

Fig. 5. Effect of ascorbate-iron lipid peroxidation using boiled and fresh microsomes on GSH concentration. GSH was added to both flasks. Open symbols represent values obtained with fresh microsomes and closed symbols represent values obtained with boiled microsomes.

associated with its oxidation to GSSG unless it is a at a very low level. In further experiments (not shown) 0.1 mM GSSG in the flask did not affect the protective lag caused by 0.1 mM GSH. Thus, the results in Fig. 5 cannot be explained by accumulation of GSSG with inhibition of the GSH effect.

Because GSH apparently was not being consumed by the protective factor, it was considered possible that GSH was stabilizing or protecting the factor against inactivation. This possibility was examined in two types of experiments. Warming the microsomes to 37°C for 1 h under air almost abolished the GSH effect on the lag (Fig. 6). The presence of 0.1 mM GSH during warming, however, partially preserved the GSH effect (Fig. 6). In the second type of experiment, addition of GSH after the incubation had been started but before lipid peroxidation had begun resulted in a shorter lag than when GSH had been present from the beginning of the incubation (Fig. 7). Thus, GSH may be protecting against heat inactivation and against free radical injury to the protein. However, these results are only suggestive of those conclusions and do not rule out other possibilities. Also they shed no light on the biochemical mechanisms involved.

Lipid peroxidation can be inhibited by free radical scavenging or by peroxide removal. Thus, either of these mechanisms could prevent the for-

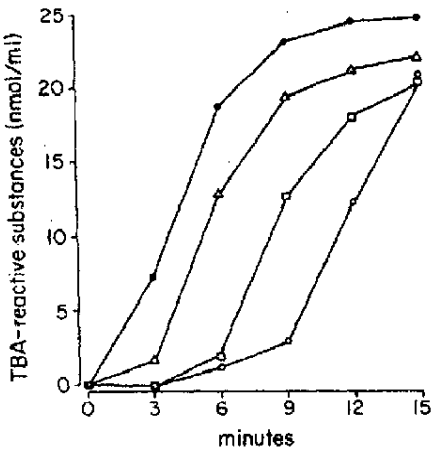

Fig. 6. GSH protection of the GSH-dependent lipid peroxidation protective protein against heat inactivation. Fresh microsomes (10 mg protein/ml) were divided into 2 aliquots. The control aliquot was kept on ice. The experimental aliquot was further divided into 2 aliquots and 0.1 mM GSH was added to one. They were both heated for 60 min at 37°C. Four incubations were done: ●———●, control microsomes with no GSH; ○———○, control microsomes with 0.1 mM GSH; △———△, microsomes heated in the absence of GSH with 0.1 mM GSH in the incubation; □———□, microsomes heated in presence of 0.1 mM GSH with 0.1 mM GSH in the incubation.

mation of thiobarbituric acid-reactive substances in the microsomal lipid peroxidation system. If free radicals are scavenged, O_2 consumption and polyunsaturated fatty acid destruction are also prevented. If peroxides are formed, however, and then destroyed by GSH, some O_2 consumption and polyunsaturated fatty acid destruction will occur even though no thiobarbituric acid-reactive substances are produced. We measured O_2 consumption in the microsomal lipid peroxidation system and correlated it with production of thiobarbituric acid-reactive substances (Fig. 8). The same ratio of O_2 consumption to thiobarbituric acid-reactive substance production was found whether GSH was present or not. This suggests that the GSH-dependent protective factor prevents lipid peroxidation by scavenging free radicals. This conclusion is also supported by Fig. 2 which indicates that the effect of GSH is exerted at a step before alteration of the polyunsaturated fatty acids occurs.

Effectiveness of GSH against NADPH microsomal lipid peroxidation

The GSH-dependent protective factor is effective against lipid peroxidation in the NADPH-driven microsomal lipid peroxidation system (Fig. 9) as well as in the ascorbate-driven system. However, GSH has little effect on the oxidation of

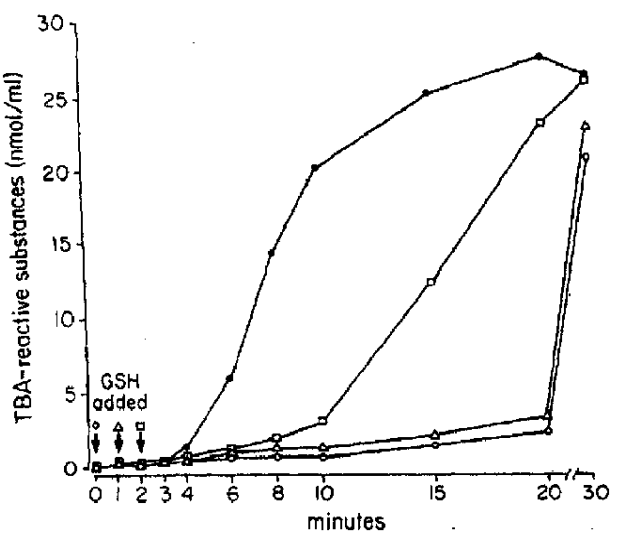

Fig. 7. Effect of delayed addition of GSH on the GSH protection against ascorbate-iron microsomal lipid peroxidation. GSH was added to the flasks at the times indicated. No GSH was added to one flask (●———●).

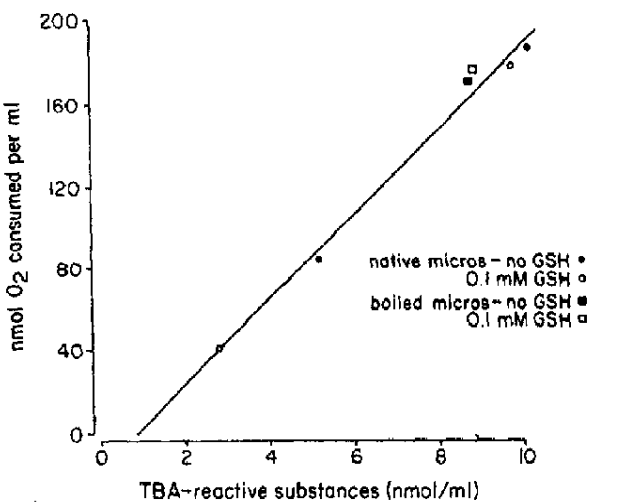

Fig. 8. Correlation of oxygen consumption with thiobarbituric acid-reactive substance production in the microsomal lipid peroxidation system: the effect of GSH. The reactions were run in the oxygen electrode and thiobarbituric acid-reactive substances were measured at various time points after rapid lipid peroxidation had begun. The line was determined by linear regression of the ascorbate system values.

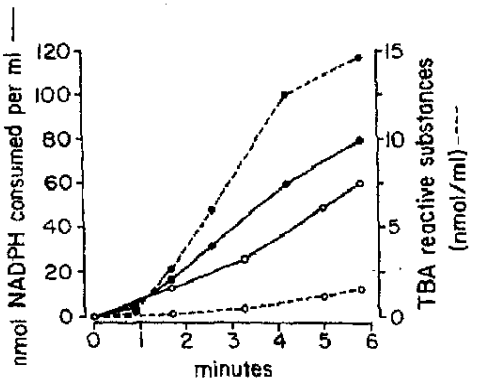

Fig. 9. Effect of GSH on NADPH oxidation and lipid peroxidation in the NADPH-microsomal lipid peroxidation system. The reaction was carried out in a 3 ml cuvette at 37°C with stirring. The NADPH consumption was monitored using the wavelength pair 340–380 nm and $E = 5.26\ mM^{-1} \cdot cm^{-1}$. ●, no GSH; ○, 0.1 mM GSH.

NADPH in this system, suggesting that free radical formation is not inhibited.

Discussion

These studies demonstrate the existence of a GSH-dependent lipid peroxidation defense mechanism in rat liver microsomes. The heat denaturation and trypsin experiments indicate that the protective factor is protein in nature and very labile in the absence of GSH. In fact, we observed loss of the factor in refrigerated or frozen microsomes over a period of days to weeks. The requirement for GSH appears to be rather specific since several other sulfhydryl compounds and GSSG could not replace GSH even when they were added at much higher concentrations.

Several observations indicate that the microsomal protective factor is distinct from cytosolic protective factors reported earlier [1–3]. First, extensive washing failed to remove the factor from the microsomal fraction. Second, our results indicate that the cytosolic factors will not function with 0.1 mM GSH and other workers have reported that the cytosolic factors require at least 1 mM GSH [14]. The microsomal protective factor functions well with 0.1 mM GSH. Finally, mercaptoethanol and dithiothreitol can replace GSH with the cytosolic factor [14], and our results indicate that these compounds have no effect with the microsomal protective factor. Thus, we conclude that the microsomal protective factor is not a contaminant from the cytosol.

The oxygen-consumption and the polyunsaturated fatty acid-consumption experiments strongly suggest that the GSH-dependent protection against lipid peroxidation is a result of free radical scavenging. In view of this it is suprising that we found no evidence of GSH oxidation, although a very low level could have escaped our detection. GSH may stabilize the protective protein rather than provide reducing equivalents for radical annihilation. Another possibility is that GSH serves as a cofactor for the protein. Thus, the question of the disposition of the radicals remains open.

It seems likely that the protective factor is an enzyme, but we have not proven that in these studies. The results could be explained by titration of free radicals against radical scavenging sites on the protein. Another potential explanation is that the protective protein catalyzes the annihilation of the free radicals either in a dismutation reaction or by utilizing an unknown substrate. Additional studies will be required to examine these possibilities.

The identity of the protective protein or proteins is not known. It is unlikely to be the selenium-dependent glutathione peroxidase because that enzyme is not found in microsomes [15,16]. GSH *S*-transferases are present in the microsomal fraction [17] and a role for them has not been excluded.

A number of reactions occurring in microsomes produce free radicals either as intermediates or final products. The membrane then requires protective mechanisms to prevent free radical injury. The microsomal GSH-dependent protein(s) described here seems eminently suited to provide such protection.

Acknowledgements

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